Induction of p53-regulated Genes and Tumor Regression in Lung Cancer Patients after Intratumoral Delivery of Adenoviral p53 (INGN 201) and Radiation Therapy[1]

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ABSTRACT

Purpose: **We designed a prospective single arm Phase II study to evaluate the feasibility and mechanisms of apoptosis induction after *Ad-p53* (*INGN 201*) gene transfer and radiation therapy in patients with non-small cell lung cancer.**

Experimental Design: **Nineteen patients with nonmetastatic non-small cell lung cancer who were not eligible for chemoradiation or surgery were treated as outpatients with radiation therapy to 60 Gy over 6 weeks in conjunction with three intratumoral injections of *Ad-p53* (*INGN 201*) on days 1, 18, and 32.**

Results: **Seventeen of 19 patients completed all planned radiation and *Ad-p53* (*INGN 201*) gene therapy as outpatients. The most common adverse events were grade 1 or 2 fevers (79%) and chills (53%). Three months after completion of therapy, pathologic biopsies of the primary tumor revealed no viable tumor (12 of 19 patients, 63%), viable tumor (3 of 19 patients, 16%), and not assessed (4 of 19 patients, 21%). Computed tomography and bronchoscopic findings at the primary injected tumor revealed complete response (1 of 19 patients, 5%), partial response (11 of 19 patients, 58%), stable disease (3 of 19 patients, 16%), progressive disease (2 of 19 patients, 11%), and not evaluable (2 of 19 patients, 11%). Quantitative reverse transcription-PCR analysis of the four *p53* related genes [*p21* (*CDKN1A*), *FAS*, *BAK*, and *MDM2*] revealed that Bak expression was increased significantly 24 h after *Ad-p53* (*INGN 201*) injection and levels of *CDKN1A* and *MDM2* expression were increased over the course of treatment.**

Conclusions: **Intratumoral injection of *Ad-p53* (*INGN 201*) in combination with radiation therapy is well tolerated and demonstrates evidence of tumor regression at the primary injected tumor. Serial biopsies of the tumor suggest that *BAK* gene expression is most closely related to *Ad-p53* (*INGN 201*) gene transfer.**

INTRODUCTION

Many genes involved in signal transduction, cell cycle control, and apoptosis have been implicated in the etiology of

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cancer (1–3) and with elucidation of the mechanisms of action of the products of these genes, many have become potential therapeutic targets (4). The tumor suppressor gene, *p53*, normally responsible for detecting damaged DNA and either directing repair or committing a cell to apoptosis (programmed cell death), is mutated or otherwise altered in >50% of human cancers, including 40–70% of NSCLCs[3]. Altered *p53* has been associated with poor prognosis in patients with many types of cancers (5–8), and several gene replacement-based therapeutic strategies for cancer are in clinical trials (4). However, the precise mechanisms of apoptosis associated with induction of tumor suppressor gene function in human cancers *in situ* have not been previously identified.

Because of the apparent links between *p53* and apoptosis and between apoptosis and radiation, we extended our previous studies of *Ad-p53* gene therapy in NSCLC and initiated a clinical trial of *Ad-p53* (INGN 201) combined with external beam ionizing radiation. We report successful gene transfer, low toxicity, and evidence of tumor regression. In addition, we examined the effects of *Ad-p53* (*INGN 201*) in combination with radiation on cell function by examining expression of several genes known to be regulated by *p53*: *p21* (*CDKN1A*), *FAS*, *BAK*, and *MDM2*. *BAK* expression, alone, was significantly increased 24 h after injection of *Ad-p53* (*INGN 201*) and thus appeared to be the marker most acutely up-regulated by *Ad-p53* (INGN 201), providing the first demonstration of the induction of an apoptotic pathway by tumor suppressor gene expression in actual human cancers.

MATERIALS AND METHODS

Protocol Approval and Data Analysis. The protocol used in this study was approved by the Biosafety and Surveillance Committees of The University of Texas M. D. Anderson Cancer Center, the Recombinant DNA Advisory Committee of the NIH, and the United States Food and Drug Administration. The authors had full access to all of the data in this study and take complete responsibility for the integrity of the data and the accuracy of the data analysis.

Gene Transfer Vector. *Ad-p53* (*INGN 201*) was supplied by Introgen Therapeutics, Inc. (Houston, TX), in frozen aliquots containing 1×10^{12} vp/ml in PBS containing 10% glycerol. Construction and generation of the vector was reported previously (9). Briefly, a replication defective adenovirus serotype 5 was constructed by replacing the viral E1 region with a *p53* expression cassette consisting of a wild-type *p53* gene flanked by the cytomegalovirus promoter and the SV40 polyadenylation signal (9).

Eligibility Criteria and Treatment Protocol. Patients enrolled in the study had histologically proven nonmetastatic NSCLC (stage I–III) with measurable disease. Patients were ineligible for chemoradiation or surgery because of significant comorbidities, age, or obstructed bronchi. Initial treatment with radiotherapy was judged to be the accepted standard of care. The presence of a *p53* mutation in the tumor was not a requirement for study entry.

Study treatment consisted of intratumoral needle injections of *Ad-p53* (*INGN 201*) on days 1, 18, and 32 of treatment in an outpatient setting. Radiation therapy was administered concurrently over 6 weeks, beginning on day 4, to a total of 60 Gy and was directed at the primary tumor and mediastinal lymph nodes if involved. Vector administration was performed by intratumoral injection of the primary tumor either through a flexible bronchoscope or by CT guided percutaneous needle as previously described (10). Tumors ≥ 4 cm in the largest diameter were injected with 10 ml divided into three separate sites, whereas tumors with a diameter of <4 cm were injected in a single site with 3 ml. *Ad-p53* doses were escalated initially in cohorts of three for the first 9 patients (3×10^{11}, 1×10^{12}, and 3×10^{12} vp of *Ad-p53*). All subsequent patients received the highest dose (3×10^{12} vp). Core biopsies were obtained from indicator lesions on days 1, 18, 19, and 32 and 3 months after treatment.

Response and Toxicity. The toxic effects of therapy were evaluated according to the National Cancer Institute's Common Toxicity Criteria (11). An independent Data Monitoring Committee whose members were not affiliated with either the University of Texas M. D. Anderson Cancer Center or the sponsor of the trial assessed response to therapy. Assessments were made of overall response (including metastatic sites) and response of the injected tumor (excluding metastatic disease). The Data Monitoring Committee used standard criteria with CT and bronchoscopic findings and not pathologic biopsies (12). Response of the primary injected tumor focused only on the primary tumor, excluding progression at metastatic sites.

Survival duration was measured from beginning of therapy to date of last follow-up or death. Time to progression for metastatic (pleural effusions, pulmonary nodules, systemic metastases) and locoregional disease (primary tumor and mediastinal or hilar lymph nodes) was defined as the time from beginning of therapy to documented progression. Patients who did not demonstrate progression were censored at the time of last follow-up.

Radiation Therapy. External radiation therapy was given by linear accelerator 18 or 6 Mv with a total dose of 60 Gy calculated at the isocenter in 30 fractions over 6 weeks without inhomogeneity correction. The margins ranged from 2 to 2.5 cm around the gross target volume.

Real-Time PCR and Reverse Transcription-PCR. Probes and primers used in this study were designed using the Primer Express software (version 1.0; Perkin-Elmer). Sequences are available upon request. To avoid amplification of contaminating residual genomic DNA, probe and primer sets for each gene were designed around the junction region of two exons so that they are mRNA-specific.

To determine the copy number of *Ad-p53* virus in each cell, viral DNA extracted from *Ad-p53* was used as an absolute standard, and the β-actin gene was used as a reference gene to count cell numbers. Briefly, calculation of *p53* virus copy number was accomplished by plotting a β-actin standard curve, using human genomic DNA (from Perkin-Elmer) as a standard (1 ng of DNA equals ~303 genome equivalents) and comparing the results of the clinical samples with the standard using

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; vp, viral particles; CT, computed tomography; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; *wt-p53*, wild-type *p53*.

β-actin probes. This resulted in the number of genomes (cells) in each sample. The number of *p53* virus copies in each sample was determined by comparing with a separate *p53*-virus standard curve (plotted using *p53*-viral DNA standard.) This value was corrected for the presence of inflammatory cells.

For quantitative real-time reverse transcription-PCR, human total RNA was used as a relative standard and human *GAPDH* gene served as an internal control for relative mRNA amount. Real-time PCR was performed in the ABI Prism 7700 Sequence Detection System according to the manufacturer's protocol.

Statistical Considerations. The purpose of this nonrandomized Phase I/II study was to evaluate the efficacy of *Ad-p53* (*INGN 201*) gene therapy as an adjunct to radiation therapy in the treatment of patients with NSCLC. The primary end point for evaluation was the local control of tumor at 3 months. The study was designed to test the null hypothesis that the 3-month local control rate is 20% *versus* the alternate hypothesis that the rate is 40% using a one-sided exact binomial test with an α level of 5%. The sample size of 49 patients provided a power of 83%. An interim analysis after 15 patients was included in the design.

Data from 17 of 19 patients enrolled in this study were analyzed. For each patient, the gene expression for *p53* and *GAPDH* were measured in duplicate at four distinct time points: time 0 = baseline, before any therapy; time 1 = on day 18 after the first *Ad-p53* (*INGN 201*) injection and after 2 weeks of radiation therapy but before the second *Ad-p53* (*INGN 201*) injection; time 2 = on day 19, 24 h after the second *Ad-p53* (*INGN 201*) injection and after 2 weeks of radiation therapy; and time 3 = at day 32, before the third *Ad-p53* (*INGN 201*) injection and after 4 weeks of radiation therapy. Ratio of the marker gene and *GAPDH* was calculated to estimate the amount of target gene expression. The coefficient of variation (defined as SD divided by the mean) was computed to estimate the precision of the duplicate experiment. Modulation of gene expression over time was assessed by comparing the ratios of the target gene between two time points. Exact binomial test was applied to test the null hypothesis of no modulation by assuming that the probability of up-regulation (or down-regulation) equals to 0.5. Two-sided *P*s were calculated. Duplicate experiments were done when adequate tissue samples were available (patients 16, 15, 13, 12, and 11 at time 0, 1, 2, and 3, respectively). The coefficient of variation ranged from 0 to 1.21 with a median of 0.2. There were 78 and 97% of the samples with coefficient of variation < 0.5 and 0.8, respectively, indicating good reproducibility between the duplicate experiments. The precision was similar among all four time points.

RESULTS

Patient and Tumor Characteristics. Nineteen patients (8 female, 11 male; median age, 74, age range, 53–91) with nonmetastatic NSCLC who were not eligible for chemoradiation or surgery were enrolled in this Phase II study (Table 1) between April 30, 1998, and May 4, 2000. The date of last follow-up was April 1, 2002, with a median follow-up of 36 months. All patients had histologically determined viable NSCLC on pretreatment tumor biopsies. Nine patients had locoregionally advanced NSCLC (5 stage IIIA and 4 stage IIIB) and were ineligible for chemoradiation because of poor performance status, age, comorbidities, or obstructed bronchi. Ten patients with stage I and II NSCLC (2 stage IA, 5 stage IB, and 3 stage IIB) were ineligible for surgery because of poor pulmonary function tests or significant comorbidities.

Patients were treated with radiation therapy to 60 Gy over 6 weeks, in conjunction with three intratumoral injections of *INGN 201* (on days 1, 18, and 32) via CT guidance (15 patients) or bronchoscopy (4 patients) administered into the primary tumor (for dose assignment see Table 1). Seventeen of 19 patients received all planned treatment, whereas 2 patients did not complete therapy because of tumor progression (patient no. 18) or early death (patient no. 6). Two additional patients did not receive tumor biopsies 3 months after completion of therapy because of tumor progression (patient no. 10) or weakness (patient no. 19). The presence of a *p53* mutation in the primary tumor was not required for entry into the study because previous studies in animal models, as well as in clinical trials, have shown no clear relationship between *p53* mutational status and response to INGN 201 treatment (10, 13–15).

Seventeen of 19 patients completed the radiation therapy according to the protocol with a total tumor dose of 60 Gy in 30 fractions (2 Gy/day) by high energy ($\geq$6 Mv) accelerated photons. The duration of the radiation therapy ranged from 39 to 50 days with the median duration as 44 days. There were no major protocol violations such as prolonged interruption of radiation therapy or administration of nonstudy anticancer therapy among the 17 patients.

Antitumoral Efficacy. Three months after completion of radiation therapy and *Ad-p53* (*INGN 201*) therapy, antitumoral efficacy was determined with CT scan evaluation (16 of 19 patients) and pathologic examination of biopsies (15 of 19 patients). Pathologic examination of biopsies 3 months after completion of therapy revealed no viable tumor in 12 patients (12 of 19, 63%) and viable tumor in 3 of 19 patients (16%). Tumors of 4 patients (4 of 19, 21%) were not biopsied because of tumor progression (patients nos. 10 and 18), early death (patient no. 6), or weakness (patient no. 19). The study was closed to additional accrual after the planned interim analysis after 19 patients.

Assessment of the primary injected tumor 3 months after completion of therapy (Table 1, Fig. 1, *A* and *B*) was performed by an external review board with CT and bronchoscopic findings and demonstrated: a CR in 1 patient (1 of 19, 5%); PR in 11 patients (11 of 19, 58%); stable disease in 3 patients (3 of 19, 16%); and PD in 2 patients (2 of 19, 11%). Two patients (2 of 19, 11%) were nonevaluable because of tumor progression (patient no. 18) or early death on treatment day 69 (patient no. 6).

Overall tumor response (including metastatic disease apparent at the time of posttreatment evaluation) was determined by an external review board, based on CT, bronchoscopic, and clinical findings. CR was seen in 1 patient (1 of 19, 11%), PR in 5 patients (5 of 19, 26%), SD in 1 patient (1 of 19, 5%), and PD in 11 patients (11 of 19, 58%). Six patients progressed locoregionally (4 primary injected tumor alone, 1 primary injected tumor, and mediastinal lymph nodes [lymph nodes irradiated but not injected with *Ad-p53* (*INGN 201*)], 1 mediastinal lymph node alone [not irradiated or injected with *Ad-p53* (*INGN 201*)]. With an intention to treat analysis and median follow-up

Table 1 Characteristics of NSCLC patients and tumors after treatment with intratumoral injection of Ad-p53 and radiation therapy

Patient no.	Ad-p53 dose (viral particles)	Age (yr)	Gender	Histology[a]	Stage	Injection site[b]	Baseline measure of primary tumor (cm^2)	Injected site response[c]	3-month biopsy[d]	Locoreg recur[e]	Distant recur[e]	Overall response[f]
1	3×10^{11}	74	Male	Squam	IIIA	RUL	4 × 4	PR	Pos	No	Yes	PD
2	3×10^{11}	78	Female	NSCLC	IIB	LUL bronchus	4 × 4	PR	Neg	No	No	PR
3	3×10^{11}	69	Male	Squam	IIB	RUL bronchus	3 × 5	CR	Neg	No	No	CR
4	1×10^{12}	73	Female	Adeno	IIIA	RLL	6 × 4	SD	Neg	Yes	Yes	PD
5	1×10^{12}	74	Male	Adeno	IIIB	LUL	8 × 5	PR	Neg	No	Yes	PD
6	1×10^{12}	72	Male	Squam	IIIB	LLL	9 × 7	NE[g]	NE[g]	No	No	PD
7	3×10^{12}	74	Female	Adeno	IB	RUL	4 × 4	PR	Neg	Yes[h]	Yes	PR
8	3×10^{12}	81	Female	Adeno	IIB	RUL	4 × 3	PR	Pos	Yes	No	PR
9	3×10^{12}	68	Male	Adeno	IB	RUL	5 × 5	SD	Pos	No	No	SD
10	3×10^{12}	53	Male	Squam	IIIA	R mainstem bronchus	5 × 7	NE[g]	NE[g]	No	Yes	PD
11	3×10^{12}	73	Male	Adeno	IIIA	LUL	5 × 5	PR	Neg	No	No	PR
12	3×10^{12}	79	Female	Squam	IA	RUL	3 × 2	PR	Neg	No	No	PR
13	3×10^{12}	91	Male	Adeno	IIIB	RUL	6 × 7	PR	Neg	No	Yes	PD
14	3×10^{12}	85	Female	Squam	IB	LLL	2 × 3	SD	Neg	No	Yes	SD
15	3×10^{12}	81	Female	Adeno	IB	RUL	3 × 2	PD[i]	Neg	No	No	PD[i]
16	3×10^{12}	67	Male	Squam	IB	RUL	8 × 5	PR	Neg	Yes[h]	Yes	PD
17	3×10^{12}	58	Female	Squam	IIIB	RUL bronchus	3 × 6	PR	Neg	Yes	Yes	PD
18[j]	3×10^{12}	62	Male	Squam	IIIB	LUL	9 × 9	PD	NE[g]	Yes	Yes	PD
19	3×10^{12}	84	Male	NSCLC	IA	RLL	2 × 3	PR	NE[g]	No	Yes	PD

[a] All tumors histologically confirmed, pretreatment, to be viable NSCLC: squam, squamous cell carcinoma; adeno, adenocarcinoma.

[b] Location of primary tumor injected with Ad-p53 (INGN 201). Tumors located in bronchus injected by bronchoscopy others by CT guidance: RUL, right upper lobe; RLL, right lower lobe; LUL, left upper lobe; LLL, left lower lobe.

[c] Response of injected primary tumor determined 3 months after completion of therapy as determined by external review board. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

[d] Pathologic biopsy of tumor obtained 3 months after completion of therapy by CT scan, core needle, or bronchoscopic biopsy.

[e] Locoregional and metastatic recurrence determined by serial bronchoscopy or CT scans. Locoregional recurrence defined as progression in primary tumor or locoregional lymph nodes (mediastinal or bronchial). Metastatic recurrence defined as progression in distant sites, including other organs, pulmonary nodules, and pleural effusions.

[f] Overall response determined 3 months after completion of therapy by external review board. Clinical progression of disease (patient no. 6).

[g] NE, not evaluable because patient died before biopsy (patients nos. 6, 10) or refused biopsy because of disease progression (patients nos. 18, 19).

[h] Mediastinal lymph nodes that were not injected with INGN 201 or in radiation portals increased in size (patient no. 16), whereas the primary tumor that was irradiated and injected with INGN 201 decreased in size. Patient no. 7 developed progression in primary tumor and mediastinal lymph nodes that were both in radiation portals.

[i] PD because CT scan showed increase in size at 3 months but probable radiation change because no tumor progression over subsequent 18 months of CT follow-up and negative pathologic biopsy at 3 months.

[j] Patient no. 18 did not complete INGN 201 and radiation therapy treatment because of clinical progression.

of 37 months, overall survival analysis by Kaplan Meier is 47% at 1 year and 26% at 3 years (Fig. 2*A*). Five patients are currently alive without evidence of disease 34–48 months after the start of treatment. Eleven patients have developed clinically diagnosed distant metastases (5 pleural effusions, 2 pulmonary nodules, 1 bone, 1 brain, 1 brain, and s.c. nodules and 1 adrenal). Median time to progression has not been reached (Fig. 2*B*) for locoregional disease and is 9.2 months for metastatic disease (Fig. 2*C*).

Adverse Events. All patients received radiation therapy and *INGN 201* gene therapy as outpatients. The most common adverse events associated with *Ad-p53* (*INGN 201*) vector administration and radiation were grade 1 or 2 fevers (79%), pain (68%), chills (53%), and pneumothoraces (37%; Table 2). All pneumothoraces were managed on an outpatient basis, with observation or percutaneous catheters. Radiation therapy was associated with primarily grade 1 or 2 esophagitis (47%), anorexia (21%), or weakness (58%). Grade 3 (severe) or grade 4 (life threatening) toxicity was noted in 6 of 19 (33%) patients and consisted of atrial arrhythmias, anemia, weakness, anorexia, pain, nausea, dyspnea, confusion, hypotension, and hallucination. The combination of *Ad-p53* (*INGN 201*) and radiation therapy did not appear to increase toxicity over what was expected with radiation therapy alone based on previous experience (16, 17). No patients discontinued radiation therapy or *Ad-p53* (*INGN 201*) because of treatment-related adverse events. In addition, no treatment related deaths were observed.

Assessment of Gene Transfer by Ad-p53 (INGN 201). The presence of *Ad-p53* vector-specific DNA and mRNA was assessed (Table 3) by quantitative real-time PCR in preinjection (day 18 after entry into the protocol) and 24 h after injection (day 19) biopsy specimens. The β-actin genome number is

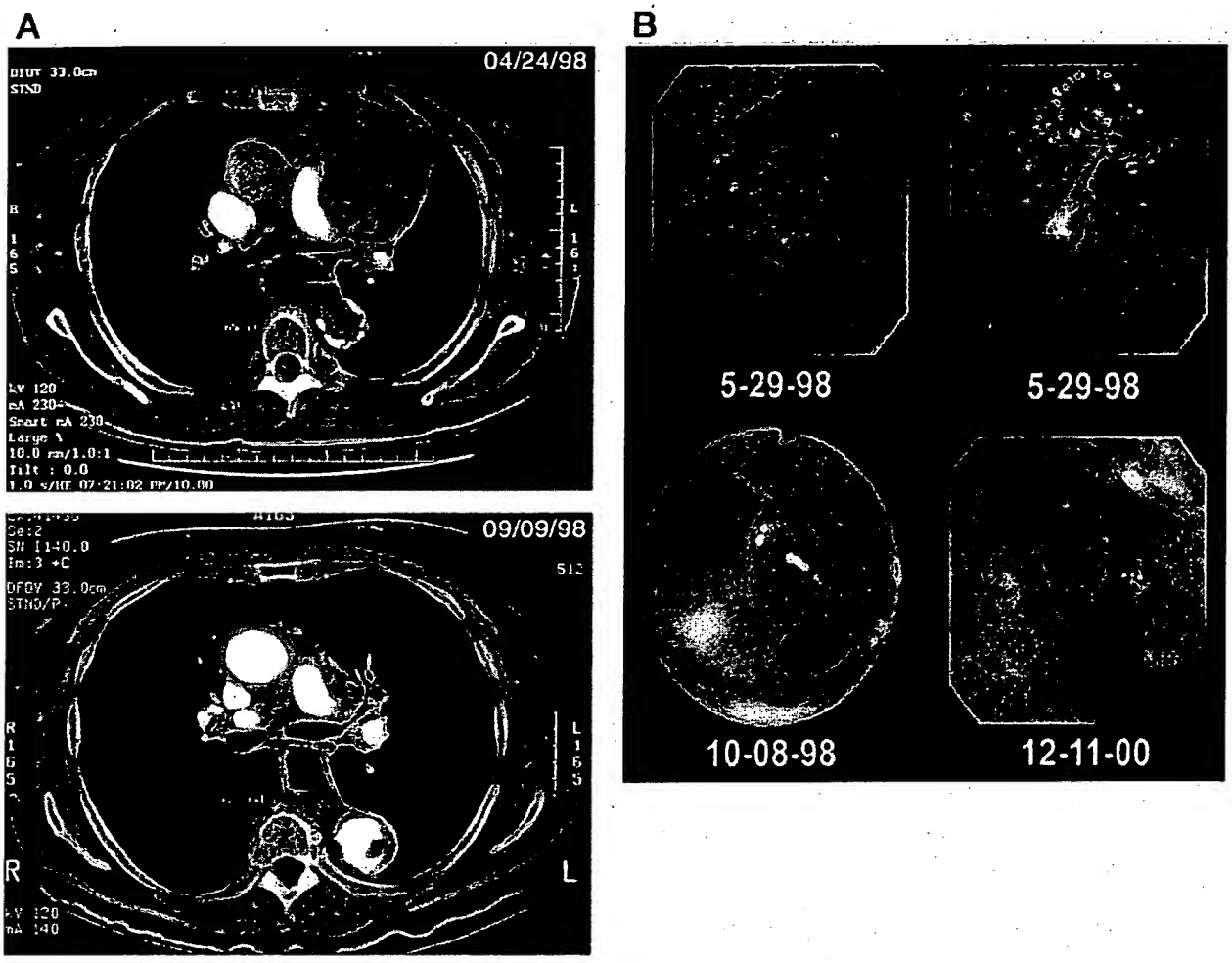

Fig. 1 *A*, patient no. 2: left upper lobe tumor unable to undergo surgery because of poor pulmonary function and cardiac disease. Patient received three injections of *Ad-p53* (3×10^{11} vp) via bronchoscope in combination with radiation therapy (60 Gy; *A*). Pathologic biopsy negative for viable tumor 3 months after completion of therapy (*B*). *B*, patient no. 3: right upper lobe tumor unable to be treated with surgery because of poor pulmonary function and ineligible for chemotherapy because of cardiac disease and obstructed bronchus (5/29/98). Patient was treated with three injections of *Ad-p53* (3×10^{11} vp) and radiation therapy (60 Gy) by bronchoscopy (5/29/98) with a CR 3 months after completion of therapy (10/8/98) and no pathologic evidence of tumor 29 months after therapy (12/11/00).

given in the table in parentheses as a positive control to show the presence of DNA in the sample. All values were corrected for the percentage of nontumor cells present in the biopsy.

Ad-p53 vector-specific DNA was detected in biopsies from 9 of 12 patients with paired biopsies (day 18 and day 19). No *Ad-p53* vector-specific DNA was detected in pretreatment biopsy specimens before the first *Ad-p53* injection (data not shown). The ratio of copies of *Ad-p53* vector DNA to copies of β-actin DNA was 0.15 or higher in 8 of 9 patients (range, 0.05–3.85) with 4 patients having a ratio >0.5. For 11 patients with adequate samples for both vector DNA and mRNA analysis, 8 showed a postinjection increase in mRNA expression associated with detectable vector DNA. Postinjection increases in *p53* mRNA were detected in 11 of 12 paired biopsies obtained 24 h after *Ad-p53* (*INGN 201*) injection, with 10 of 11 increasing 3-fold or greater ($P < 0.005$). Comparison of *Ad-p53* mRNA levels in days 18, 19, and 32 biopsy specimens to pretreatment biopsies also showed a highly statistically significant increase (data not shown; $P < 0.005$). Preinjection biopsies that were negative for *p53* protein expression by immunohistochemistry were stained for *p53* protein expression after *Ad-p53* (*INGN 201*) injection. Staining results confirmed that the *p53* protein was expressed in the posttreatment samples in the nuclei of cancer cells (data not shown).

Effect of *Ad-p53* (*INGN 201*) Gene Transfer on mRNA Expression of *p53*-regulated Genes. Previous *in vitro* experiments in human NSCLC cell lines identified four genes [*p21* (*CDKN1A*), *MDM2*, *FAS*, and *BAK*] that showed the greatest increase in mRNA expression after induction of *p53* overexpression with *Ad-p53* (data not shown). Therefore, in the current study, changes in mRNA levels for these four markers were determined at various time points before and during treatment using reverse transcriptase real-time PCR (Table 4). The study was controlled by obtaining a pretreatment biopsy under the

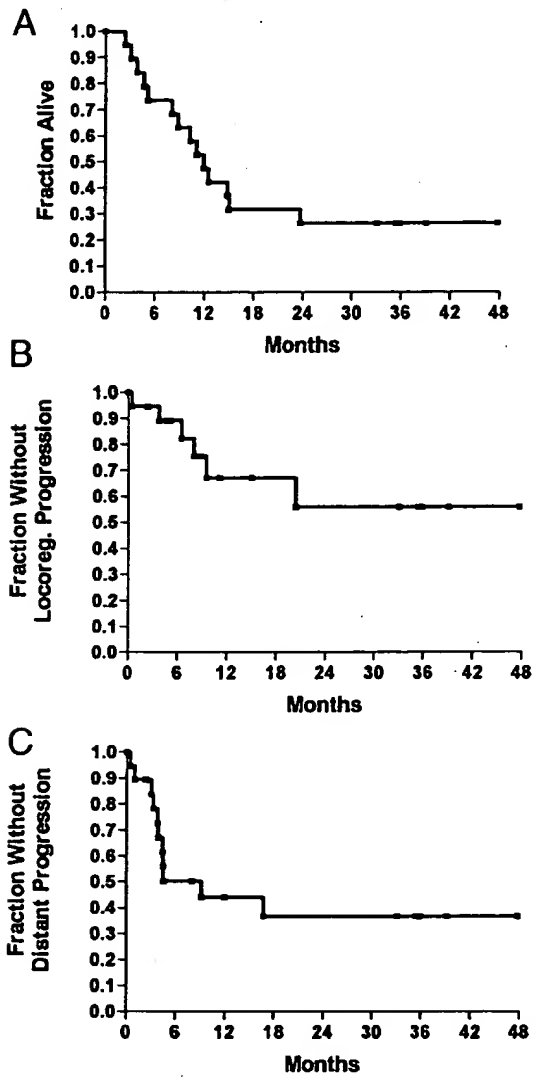

Fig. 2 *A*, overall survival of all patients (n = 19) entered on trial with *Ad-p53* (*INGN 201*) and radiation therapy. *B*, locoregional time to progression of all patients (n = 19) entered on trial with Ad-p53 (*INGN 201*) and radiation therapy. *C*, metastatic time to progression of all patients (n = 19) entered on trial with Ad-p53 (*INGN 201*) and radiation therapy.

same conditions as the posttreatment biopsy. The inclusion of a time point during the radiation treatment allowed for a biopsy to be performed immediately before and 24 h after *Ad-p53* injection, thus allowing determination of the effects of the *Ad-p53* on mRNA expression during treatment. An increase in mRNA expression was defined as a ratio >2 compared with GAPDH; a decrease was defined as a ratio <0.5; any value between 0.5 and 2 was considered no change. The exact binomial two-sided test was used to test the null hypothesis of no modulation between time points. The time intervals in Table 4 refer to: (*a*) change between day 18 (immediately before injection) and day 19 [24 h after *Ad-p53* (*INGN 201*) injection]; and (*b*) change between day 0 (before initiation of all treatment) and days 18, 19, and 32 (days after initiation of treatment).

For *p21* (*CDKN1A*) mRNA, increases of statistical significance were noted 24 h after *Ad-P53* injection (time interval 1, borderline, P = 0.07) and during treatment, as compared with the pretreatment biopsy (time interval 2, P = 0.02). In the case of *MDM2* mRNA, increases of statistical significance were noted during treatment compared with the pretreatment biopsy (time interval 2, P = 0.04). Levels of *FAS* mRNA did not show statistically significant changes during treatment. BAK mRNA expression increased significantly 24 h after injection of *Ad-p53*(INGN 201; time interval 1, P = 0.04) and thus appeared to be the marker most acutely up-regulated by *Ad-p53* (*INGN 201*) injection rather than *Ad-p53* (*INGN 201*) and radiation therapy (time interval 2, P = 0.80). There were too few specimens to find a statistical correlation with gene up-regulation and clinical outcome.

DISCUSSION

Conventional radiation and sequential chemoradiation strategies provide poor locoregional control of NSCLC, with only 15–20% local control at 1–2 years (16, 17). In an effort to determine whether *Ad-p53* (*INGN 201*) could enhance locoregional control, this trial was designed to evaluate not only radiographic responses but also pathologic biopsies 3 months after completion of therapy. A previous study by Le Chevalier *et al.* (16) resulted in a negative pathologic biopsy rate 3 months after completion of radiation or sequential chemoradiation of only 17–20%. Although our study cannot be compared directly with this study, the high number of pathologic negative biopsies (63%, intention to treat) and the large number of radiological responses at the primary tumor site [60% major response (PR or CR), intention to treat] in a patient population that was unable to tolerate chemoradiation or surgery is encouraging. Because survival in locoregionally advanced NSCLC is also dependent on control of metastatic disease, Phase III randomized studies will be necessary to determine whether the potential improvement in locoregional control achieved by *Ad-p53* (*INGN 201*) and radiation therapy can translate into improved overall survival. Our study did, in fact, demonstrate a high metastatic failure rate (Fig. 2*C*), which may have been expected because chemotherapy could not be administered to these high-risk patients. In future studies, we plan to address metastatic relapse by adding chemotherapy to the combination of *Ad-p53* (*INGN 201*) and radiation therapy.

It is encouraging that strategies designed to improve locoregional control in locoregionally advanced NSCLC such as concurrent chemoradiation or fractionated radiation therapy have led to improved survival (17–19). The Japanese Clinical Oncology Group and the Radiation Therapy Oncology Group recently reported improved survival in locoregionally advanced NSCLC when concurrent chemoradiation rather than sequential chemoradiation was used, presumably because of the radiation sensitizing effect of concurrent chemotherapy (19–21), although toxicity appeared increased with concurrent chemotherapy. Additionally, subset analysis of these studies have demonstrated that concurrent chemoradiation might not be as effective in elderly or poor performance status patients, in part, because of increased toxicity (22). These observations suggest a therapeutic window for *Ad-p53* (*INGN 201*) and radiation therapy

Table 2 Highest grade adverse event (AE) observed in 19 patients during *Ad-p53* (INGN 201) and radiation therapy

Adverse events[a]	Grade 1[b,c]	Grade 2[b,c]	Grade 3[b,c]	Grade 4[b,c]	Total patients with AE[d]
Fever	2 (11)	13 (68)	0	0	15 (79)
Pain	7 (37)	5 (26)	1 (5)	0	13 (68)
Weakness	4 (21)	4 (21)	3 (16)	0	11 (58)
Nausea	9 (47)	0	1 (5)	0	10 (53)
Chills	7 (37)	3 (16)	0	0	10 (53)
Esophagitis	6 (32)	3 (16)	0	0	9 (47)
Dyspnea	6 (32)	1 (5)	1 (5)	0	8 (42)
Vomiting	5 (26)	3 (19)	0	0	8 (42)
Pneumothorax	4 (21)	3 (16)	0	0	7 (37)
Arrhythmia	5 (26)	0	0	2 (11)	7 (37)
Hemoptysis	4 (21)	2 (11)	0	0	6 (32)
Anorexia	3 (16)	0	1 (5)	0	4 (21)
Hypotension	0	1 (5)	1 (5)	0	2 (11)
Anemia	0	0	2 (11)	0	2 (11)
Confusion	0	0	1 (5)	0	1 (5)
Hallucination	0	0	1 (5)	0	1 (5)

[a] Adverse events listed as descriptive or verbatim term from medical records, not otherwise coded.
[b] Toxicity defined by National Cancer Institute common toxicity criteria (Grade 1–4); percentage of patients with this level of toxicity in parentheses.
[c] Highest grade toxicity associated with *Ad-p53* (INGN 201) and radiation therapy treatment; percentage of patients with this level of toxicity in parentheses.
[d] Total number of patients treated with *Ad-p53* (INGN 201) and radiation therapy with AE.

Table 3 Detection of *Ad-P53* vector-specific DNA and mRNA in biopsy specimens by real-time PCR

	Ad-p53 DNA PCR *Ad-p53* copy number: β-actin genome[a] (copies of β-actin genome)		*Ad-p53* mRNA reverse transcriptase PCR[a] *p53* mRNA: GAPDH mRNA	
Patient no.	Preinjection[b]	Postinjection[c]	Preinjection	Postinjection
1	neg[d] (68)	neg (112)	218	5308
2	neg (149)	3.85 (92)	<1	23280
3	neg (71)	3.06 (60)	25	81
4	na[e]	na	na	na
5	neg (94)	neg (267)	176	766
6	neg (334)	0.05 (458)	35	170502
7	neg (17)	0.71 (73)	167	11365
8	neg (347)	0.39 (220)	20	33
9	neg (1221)	neg (1666)	<1	2214
10	neg (285)	0.19 (611)	<1	94
11	neg (200)	0.24 (219)	18	220
12	na	na	na	na
13	neg (194)[f]	2.76 (248)	7	701
14	0.16 (408)	neg (421)	na	na
15	na	na	na	na
16	na	na	113	78
17	na	na	na	na
18	na	na	na	na
19	na	na	na	na

[a] Values represent the mean of duplicate samples.
[b] Day 18.
[c] Day 19.
[d] neg, not detectable in biopsy; sensitivity of the quantitative DNA PCR is one copy as determined by standard curves; for the reverse transcription-PCR, the sensitivity is between 10 and 100 copies by quantitation in run-off transcription assays (data not shown).
[e] na, biopsy not available.
[f] Pretreatment biopsy was obtained before day 1 injection.

because this novel strategy demonstrated no dose-limiting toxicity with concurrent use and was limited only by manufacturing considerations. Although these patients were often ineligible for chemoradiation because of age or significant comorbidities, only 34% of the patients suffered a grade 3 or higher adverse event, and all patients were treated as outpatients. In the future, *Ad-p53* (*INGN 201*) may, in combination with chemoradiation, provide enhanced survival by increasing locoregional control without increasing toxicity.

Another major goal of this study was to determine the molecular mechanism by which *Ad-p53* (*INGN 201*) and radiation therapy-induced cell killing. We therefore performed mul-

Table 4 Changes in mRNA levels of marker genes at various time points before and during treatment

mRNA	Time interval[a]	Patients with increased[b] mRNA levels	Patients with decreased[c] mRNA levels	*P* (two-sided)
P21 (CDKN1A)	1	7	1	0.07
	2	11	2	0.02
MDM2	1	4	6	0.75
	2	8	1	0.04[d]
FAS	1	5	6	~1.00
	2	5	3	0.73
BAK	1	8	1	0.04[d]
	2	9	7	0.80

[a] Time interval 1, interval between day 18 [immediately before injection of *Ad-P53* (INGN 201)] and day 19 [24 h after *Ad-p53* (INGN 201) injection]; time interval 2 = change between day 0 (before initiation of any treatment) and days 18, 19, and 32 (days after initiation of treatment).

[b] Two-fold or greater increase over pretreatment value.

[c] Fifty percent or greater decrease from pretreatment value; patient samples not classified as either an increase or decrease are considered unchanged or not available for analysis from a total of 16 possible specimens.

[d] Statistically significant, $P < 0.05$.

tiple biopsies throughout the study to evaluate the time course of the induction of several apoptosis-related genes and their relationship to *p53* gene transfer and radiation therapy. We demonstrated for the first time in human cancers *in situ*, the induction of expression of several genes closely linked to *p53*, including *MDM2*, *p21* (*CDKN1A*), and *BAK*. Our study showed that, although *p21* (*CDKN1A*) and *MDM2* appeared to be modestly up-regulated in tumors injected with *Ad-p53* (*INGN 201*), it was the proapoptotic gene *BAK* that showed significant up-regulation within 24 h of *Ad-p53* (*INGN 201*) injection. Because of the study design, it remains possible that radiation alone had an effect on the marker genes independent of *Ad-p53* (*INGN 201*) administration. A study by Bishay *et al.* (23), however, showed that *BAK* mRNA remains at a constant level in cells with endogenous *wt-p53* after radiation. Thus, our observation of an increase in *BAK* links this specifically to the forced overexpression of *p53* by *Ad-p53* (INGN 201) gene transfer. Previously, Pearson *et al.* (24) showed up-regulation of *BAK* protein expression in lung cancer cell lines in response to forced overexpression of p53. Bishay *et al.* (23) also showed increased expression of *p21* (*CDKN1A*) in B lymphoblastoid cells, suggesting that radiation in the presence of *wt-p53* can induce *p21* (*CDKN1A*) expression. However, in our study, this cannot be specifically attributed to forced overexpression of *p53* because radiation can achieve this with low levels of *wt-p53*. In lung tumors with nonfunctional *wt-p53*, which likely includes most of tumors in our study, the restoration of *wt-p53* function probably contributed to the increases in *p21* (*CDKN1A*) expression.

The role of these genes in mediating tumor regression and apoptosis in patients will require further study. However, previous studies have shown conclusively that forced overexpression of *p53* by both retroviral and adenoviral vectors is associated with marked increases in the apoptotic fraction of NSCLC cells in biopsies taken 72 h after injection of *Ad-p53* as shown by terminal deoxynucleotide transferase-mediated biotin UTP nick-end labeling staining (10, 13, 25). The up-regulation of the proapoptotic gene *BAK* may, in part, be responsible for this effect, although this study cannot determine whether *Ad-p53* was responsible because radiation was administered simultaneously. Although many genes may be under the regulatory control of *p53*, we initially screened several NSCLC cell lines for those apoptosis-associated genes most strongly up-regulated by forced *p53* overexpression. Thus, although our study does not include all known *p53* regulated genes, it provides important confirmation of the cell culture findings and a methodology for future studies.

Future clinical trials will explore the combination of *Ad-p53* (*INGN 201*) gene transfer and chemoradiation to address both metastatic and locoregional disease. The antitumoral potential of these strategies is supported by preclinical data that suggests the combination of all three treatments (*Ad-p53*, chemotherapy, and radiation therapy) is synergistic and may lead to enhanced antitumoral activity without increased toxicity.[4] This study has been an important foundation for future studies because it provides a molecular mechanism for the enhanced antitumoral activity with sequential tumor biopsies and quantitative analysis of *p53*-regulated genes. It confirms that after *Ad-p53* (*INGN 201*) gene transfer and radiation therapy, *wt-p53* gene expression increases dramatically with subsequent induction of *BAK*, *MDM2*, and *p21* (*CDKN1A*). In the future, these molecular markers may help clinicians to identify those patients most likely to respond to *Ad-p53* (*INGN 201*) gene transfer strategies.

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REFERENCES

1. Weinberg, R. A. Oncogenes and tumor suppressor genes. CA—Cancer J. Clin., *44:* 160–170, 1994.

2. Klein, G. The approaching era of the tumor suppressor gene. Science (Wash. DC), *239:* 1539–1545, 1987.

3. Sager, R. Tumor suppressor genes: the puzzle and the promise. Science (Wash. DC), *246:* 1406–1412, 1989.

[4] Submitted for publication.

4. Roth, J. A., and Cristiano, R. J. Gene therapy for cancer: what have we done and where are we going? J. Natl. Cancer Inst. (Bethesda), *89:* 21–39, 1997.

5. Isobe, T., Hiyama, K., Yoshida, Y., Fujiwara, Y., and Yamakido, M. Prognostic significance of *p53* and *ras* gene abnormalities in lung adenocarcinoma patients with stage I disease after curative resection. Jpn. J. Cancer Res., *85:* 1240–1246, 1994.

6. Thor, A. D., Moore, D. H., Edgerton, S. M., Kawasaki, E. S., Reehaus, E., Lynch, H. T., Marcus, J. N., Schwartz, L., Chen, L. C., and Mayall, B. N. Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. J. Natl. Cancer Inst. (Bethesda), *84:* 845–855, 1992.

7. Quinlan, D. C., Davidson, A. G., Summers, C. L., Warren, H. E., and Doshi, H. M. Accumulation of p53 protein correlates with a poor prognosis in human lung cancer. Cancer Res., *52:* 4828–4831, 1992.

8. Hamada, M., Fujiwara, T., Hizuta, A., Gochi, A., Naomoto, Y., Takakura, K., Roth, J. A., Tnaaka, N., and Orita, K. The *p53* gene is a potent determinant of chemosensitivity and radiosensitivity in gastric and colorectal cancers. J. Cancer Res. Clin. Oncol., *122:* 360–365, 1996.

9. Zhang, W. W., Fang, X., Branch, C. D., Mazur, W., French, B. A., and Roth, J. A. Generation and identification of recombinant adenovirus by lipsome-mediated transfection and PCR analysis. Biotechniques, *15:* 868–872, 1993.

10. Swisher, S. G., Roth, J. A., Nemunaitis, J., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Connors, D. G., el Naggar, A. K., Fossella, F., Glisson, B. S., Hong, W. K., Khuri, F. R., Kurie, J. M., Lee, J. J., Lee, J. S., Mack, M., Merritt, J. A., Nguyen, D. M., Nesbitt, J. C., Perez-Soler, R., Pisters, K. M. W., Putnam, J. B., Richli, W. R., Savin, M., Schrump, D. S., Shin, D. M., Skulkin, A., Walsh, G. L., Wait, J., Weill, D., and Waugh, M. K. A. Adenovirus-mediated p53 gene transfer in advanced non-small cell lung cancer. J. Natl. Cancer Inst. (Bethesda), *91:* 763–771, 1999.

11. Ajani, J. A., Welch, S. R., Raber, M. N., Fields, W. S., and Krakoff, I. H. Comprehensive criteria for assessing therapy-induced toxicity. Cancer Investig., *8:* 147–159, 1990.

12. Roth, J. A. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. Nat. Med., *2:* 985–991, 1996.

13. Nemunaitis, J., Swisher, S. G., Timmons, T., Connors, D., Mack, M., Doerksen, L., Weill, D., Wait, J., Lawrence, D. D., Kemp, B. L., Fossella, F., Glisson, B. S., Hong, W. K., Khuri, F. R., Kurie, J. M., Lee, J. J., Lee, J. S., Nguyen, D. M., Nesbitt, J. C., Perez-Soler, R., Pisters, K. M. W., Putnam, J. B., Richli, W. R., Shin, D. M., Walsh, G. L., Merritt, J., and Roth, J. A. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small cell lung cancer. J. Clin. Oncol., *18:* 609–622, 2000.

14. Clayman, G. L., el-Naggar, A. K., Roth, J. A., Zhang, W. W., Goepfert, H., Taylor, D. L., and Liu, T-J. *In vivo* molecular therapy with *p53* adenovirus for microscopic residual head and neck squamous carcinoma. Cancer Res., *55:* 1–6, 1995.

15. Clayman, G. L., el-Naggar, A. K., Lippman, S. M., Henderson, Y. C., Frederick, M., Merritt, J. A., Zumstein, L. A., Timmons, T. M., Lui, T-J., Ginsberg, L., Roth, J. A., Hong, W. K., Bruso, P., and Geopfert, H. Adenovirus-mediated p53 gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. J. Clin. Oncol., *16:* 2221–2232, 1998.

16. Le Chevalier, T., Arriagada, R., Quoix, E., Ruffie, P., Martin, M., Tarayre, M., Lacombe-Terrier, M-J., Douillard, J-Y., and Laplanche, A. Radiotherapy alone versus combined chemotherapy and radiotherapy in non-resectable non-small cell lung cancer: first analysis of a randomized trial in 353 patients. J. Natl. Cancer Inst. (Bethesda), *83:* 417–423, 1991.

17. Schaake-Koning, C., van den Boggert, W., Dalesio, O., Festen, J., Hoogenhout, J., van Houtte, P., Kirkpatrick, A., Koolen, M., Maat, B., Nijs, A., Renaud, A., Rodriqus, P., Schuster-Vittenhoeue, L., Sculier, J-P., van Zandwijk, N., and Bartelink, H. Effects of concomitant cisplatin and radiotherapy on inoperable non-small cell lung cancer. N. Engl. J. Med., *326:* 524–530, 1992.

18. Saunders, M., Dische, B. A., Harvey, A., Gibson, D., and Parmar, M. Continuous hyperfractionated accelerated radiotherapy (CHART) versus conventional radiotherapy in non-small cell lung cancer: a randomised multicentre trial. Lancet, *350:* 161–165, 1997.

19. Furuse, K., Fukuoka, M., Kawahara, M., Nishikawa, H., Takada, Y., Kadoh, S., Katagami, N., and Ariyoshi, Y. Phase III study of concurrent versus sequential thoracic radiotherapy in combination with mitomycin, vindesine, and cisplatin in unresectable stage III non-small cell lung cancer. J. Clin. Oncol., *17:* 2692–2699, 1999.

20. Komaki, R., Scott, C., Ettinger, D., Lee, J. S., Fossella, F. V., Curran, W., Evans, R. F., Rubin, P., and Byhardt, R. W. Randomized study of chemotherapy/radiation therapy combinations for favorable patients with locally advanced inoperable non-small cell lung cancer: Radiation Therapy Oncology Group (RTOG 9410). Int. J. Radiat. Oncol. Biol. Phys., *38:* 149–155, 2000.

21. Curran, W. J., Scott, C., Langer, C., Komaki, R., Lee, J. S., Hauser, S., Movsas, B., Wasserman, T. H., Rosenthal, S., Byhardt, R., Sause, W., and Cox, J. Phase III comparison of sequential versus concurrent chemoradiation for PTS with unresected stage III non-small cell lung cancer (NSCKC): initial report of radiation therapy oncology group (RTOG) 9410. Am. Soc. Clin. Oncol., *19:* 484a, 2000.

22. Movsas, B., Scott. C., Sause, W., Byhardt, R., Komaki, R., Cox, J., Johnson, D., Lawton, C. M., Dar, A. R., Wasserman, T., Roach, M., Lee, J. S., and Andras, E. The benefit of treatment intensification is age and histology-dependent in patients with locally advanced non-small cell lung cancer (NSCLC): a quality-adjusted survival analysis of radiation therapy oncology group (RTOG) chemoradiation studies. Int. J. Radiat. Oncol. Biol. Phys., *45:* 1143–1149, 1999.

23. Bishay, K., Ory, K., Lebeau, J., Oliver, M. F., and Chevillard, S. DNA damage-related gene expression as biomarkers to assess cellular response after γ irradiation of a human lymphoblastoid cell line. Oncogene, *19:* 916–923, 2000.

24. Pearson, A. S., Spitz, F. R., Swisher, S. G., Kataoka, M., Sarkiss, M. G., Meyn, R. E., McDonnell, T. J., Cristiano, R. J., and Roth, J. A. Up-regulation of the proapoptotic mediators bax and bak after adenovirus-mediated *p53* gene transfer in lung cancer cells. Clin. Cancer Res., *6:* 887–890, 2000.

25. Roth, J. A. Retrovirus-mediated p53 gene therapy. Nat. Med., *2:* 1163, 1996.